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Paxillus albidulus, P. ammoniavirescens, and *P. validus* revisited

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Abstract—Comparison of nrITS sequences of the type specimens of *Paxillus ammoniavirescens* and *P. validus* show them to be identical. The name *Paxillus ammoniavirescens* was published in 'Spring 1999' [northern hemisphere] and that of *Paxillus validus* in August 1999, making *P. ammoniavirescens* the correct name for this species. A variant without pigments fits into *P. involutus*, suggesting that *P. albidulus* might be an albino variant of any species in the *P. involutus* complex. *Paxillus ammoniavirescens* is widespread in Europe and has been introduced in the southern hemisphere; *P. obscurosporus* is known from China and Europe; and the two taxa in the *P. involutus* clade are reported from Europe and from North America.

Keywords—*Paxillaceae*, phylogenetic species, pigment-free variants

Introduction

The genus *Paxillus* Fr. is widely distributed in the northern hemisphere, forms ectomycorrhiza with a range of host trees, is extensively used in ectomycorrhizal research (e.g. Ek 1997, Leake et al. 2001, Prendergast-Miller et al. 2011, Wilkinson et al. 2010), and is a nightmare for species recognition. There are two main species clusters — *P. rubicundulus* P.D. Orton and allies, associating with *Alnus* species, and the *P. involutus* complex, associating with a range of host trees (*Pinaceae, Fagaceae, Salicaceae*, *Corylaceae*, *Tiliaceae,* etc.). Hedh et al. (2008), who recently addressed species concepts of the European taxa in the *P. involutus* complex, recognized four species based on five gene genealogies and nrITS sequence analyses. Three of the four phylogenetic species correspond with the intercompatibility groups identified by Fries (1985). Hedh et al. (2008) recognized *Paxillus involutus* (Batsch) Fr., *P. validus*, *P. obscurosporus* C. Hahn, and a fourth species, sister to *P. involutus,* which they refrained from naming.

Table 1. Overview of *Paxillus* collections.

Species	Collection (herbarium)	Locality	Habitat	Collection date	Genbank nr ITS
ammoniavirescens	A.E. Hills 2004209 (K)	U.K.: England, Kent, Bean, Beacon Wood Country Park	in mixed forest	21 Oct. 2004	JN661711
	A.E. Hills 2005047 (K)	U.K.: England, Berkshire, Silwood Park near Ascot	with *Quercus robur*	8 Aug. 2005	JN661712
	A.E. Hills 2005059 (K)	U.K.: England, Hampshire, New Forest, Cadnam Cricket Pitch area	under *Salix caprea*	20 Sept. 2005	JN661713
	A.E. Hills 2005086 (K)	U.K.: England, Oxfordshire, Oxford, Milham Ford School	in parkland under *Tilia cordata*	30 Sept. 2005	JN661714
	A.E. Hills 2007039 (K)	France: Dept. Alpes Maritimes, Esterel-Les Espagnols	under *Alnus*	16 Oct. 2007	JN661715
	A.E. Hills 2007046 (K)	France: Dept. Alpes Maritimes, Esterel-Les Espagnols	under *Salix*	16 Oct. 2007	JN661716
	A.E. Hills 2007045 (K)	France: Dept. Alpes Maritimes, Esterel-Les Espagnols	with *Quercus suber* and *Populus*	16 Oct. 2007	JN661717
	P. Dessi (IB1997_0980, TYPE)	Italy: Sardinia, prov. Cagliari, Serramanna	—	21 Nov. 1997	JN661718
	M. Contu (UC)	Italy: Sardinia, prov. Olbia-Tempio, Olbia, Park "Fausto Noce"	near *Populus*	10 Dec. 2006	JN661719
involutus s. str. I	A.E. Hills 2005067 (K)	U.K.: England, Oxfordshire, North Leigh Common	with *Betula pendula*	24 Sept. 2009	JN661721
	E.C. Vellinga 4037 (TENN64458)	U.S.A.: TN, Great Smoky Mountains NP, Polk Co., Fork Ridge Trail	with *Picea*	3 Aug. 2009	JN661722
	M. Kuo 09130703 (UC)	U.S.A.: MI, Emmet Co., The Headlands.	—	—	JN661723
	S. Kelly (UC)	U.K.: England, Hampshire, New Forest, near Tricket Cross	—	5. Sept. 2009	JN661724
involutus II	D. Deshazer DD535 (UC)	U.S.A.: CA, Sonoma Co., Sebastopol, 406 Pleasant Hill Rd	with *Corylus* and *Quercus*	4 Dec. 2006	JN661725
	E.C. Vellinga 3211b (UC)	U.S.A.: CA, Alameda Co., Berkeley	with *Betula*	2004	JN661726
	E.C. Vellinga 3336 (UC)	U.S.A.: CA, Alameda Co., Berkeley	with *Betula*	Dec. 2005	JN661727
	E.C. Vellinga s.n. (UC)	U.S.A.: WA, King Co., Fauntleroy, Lincoln Park	with *Betula*	10 Oct. 2008	JN661728

Two other species have been described from Europe, *P. albidulus* Šutara (Šutara 1992), a predominantly white species, and *P. ammoniavirescens* (Dessì & Contu 1999), characterized by a strong green reaction of the pileus surface with ammonia. As these species were not included in the study by Hedh et al. (2008), their identity and relationship to the phylogenetic species remained unresolved.

Here, we investigate the identity of *P. ammoniavirescens* and the phylogenetic placement of taxa without pigments using nrITS sequence data and phylogenetic methods.

Material & methods

Material—Type material of *P. ammoniavirescens*, additional collections of *P. ammoniavirescens* from the type locality, various *Paxillus* collections from the UK and France, and *Paxillus* collections growing with introduced *Betula* in western North America were used for analyses (Table I).

DNA extraction to sequencing—DNA was extracted from dried material using a Qiagen DNeasy® Blood and Tissue kit (Qiagen, Valencia, CA, USA). PCR was done under standard conditions using the following primers: ITS-1F and ITS-4 for nrITS1, 5.8S and nrITS2 (Gardes & Bruns 1993). PCR reactions were performed with an MJ PTC-100™ thermocycler (Applied Biosystems, Foster City, CA, USA) or an Eppendorf Mastercycler® gradient (Eppendorf, North America, Inc.,Westbury, NY, USA). PCR products were cleaned using 0.5 µl of ExoSAP IT (USB Corp, Cleveland, OH, USA) per reaction and cycled at 37°C for 45 min, followed by 80°C for 15 min. Sequencing was performed using Big Dye chemistry, with the same primers as for PCR, and an ABI PRISM 3100 Genetic Analyzer (both from Applied Biosystems, Foster City, CA, USA). Sequences were edited and contigs assembled using Sequencher 4.2.2 (Gene Codes Corporation, Ann Arbor, MI, USA). All newly produced sequences were deposited in GenBank (see table I).

Phylogenetic methods—nrITS sequences of the European species in the *P. involutus* species complex available in GenBank and the UNITE database, from vouchered specimens and from environmental samples, were downloaded and added to the database. The sequences were aligned with the program MAFFT version 6 (Katoh et al. 2002; Katoh & Toh 2008), using default settings. The database was analyzed by a maximum likelihood (ML) method using RAxML version 7.2.3 (Stamatakis et al. 2008). *Paxillus rubicundulus* was chosen as outgroup.

Results

The four phylogenetic European species recognized by Hedh et al. (2008) are also recovered in our nrITS sequence analyses (fig. 1), with high bootstrap support for *P. validus*/*P. ammoniavirescens* (95%), *P. involutus* I (100%), and *P. involutus* II (100%). The type collection of *P. ammoniavirescens* and the type collection of *P. validus* have identical ITS sequences. Collections from England

and France with sequences newly generated for this study also belong to *P. ammoniavirescens/P. validus*, plus one collection from New Zealand. Sister to the *P. ammoniavirescens/P. validus* clade is *P. obscurosporus*, with collections from all over Europe, and one from China. The other two taxa represent *P. involutus* clade I, with specimens in Europe and North America (Wisconsin, Tennessee, British Columbia, Alaska), and *P. involutus* clade II, with representatives in Europe and in western North America where they grow with introduced *Betula*. *Paxillus involutus* I includes a specimen from England that lacks pigments.

Discussion

Hedh et al. (2008) used five genes, actA, gpiA, hydA, rabA, and ss-tubA, for phylogenetic species recognition in the *P. involutus* group, but concluded that nrITS on its own discriminated the four phylogenetic species recovered from the multi-gene analyses. Hence, for this study, nrITS was used to assign specimens to species. However, nrITS sequences alone are not sufficient to differentiate the North American species *P. vernalis* Watling from *P. validus* (Hedh et al. 2008), nor western North American collections that are very close to *P. obscurosporus* and grow with native and introduced tree species in native and anthropogenic habitats (data not shown).

The nrITS sequences of the type collections of *P. ammoniavirescens* (Genbank JN661718) and *P. validus* (Genbank EU084666) are identical. The two original descriptions emphasize different characters, e.g. Hahn & Agerer (1999) described the relatively big crystals on the rhizomorphs of *P. validus*, whereas Dessì & Contu (1999) did not comment on this character. The reactions of the various parts of the basidiocarps with a set of chemicals is given in both articles, with slight differences. Dessì & Contu (1999) emphasized the green reaction of the pileipellis in ammonia, whereas Hahn & Agerer described this reaction as 'schwarz überhaucht;' this difference in reaction might be caused by differences in age of the basidiocarp or by different concentrations of the reagent, but neither set of authors provided that information. For KOH different concentrations were used: 15% by Hahn & Agerer (1999), 5% by Dessì & Contu (1999). In both descriptions the pileus is introduced as ribbed, the two species are similar in general stature, and the spore sizes and spore print colour of the two are comparable. Thus we conclude here, based on the molecular evidence, that the two taxa are conspecific. The next question is which name was validly published first. Both names were published in 1999. *Paxillus validus*

Fig. 1. nrITS phylogeny of European *Paxillus* species inferred by maximum likelihood (ML) analysis. Numbers at internodes refer to confidence estimates based on 100 rapid ML bootstraps. Type collections are in bold; * indicates newly created sequences and '*white' indicates the variant without pigment in the pileus. *Paxillus rubicundulus* is used as outgroup.

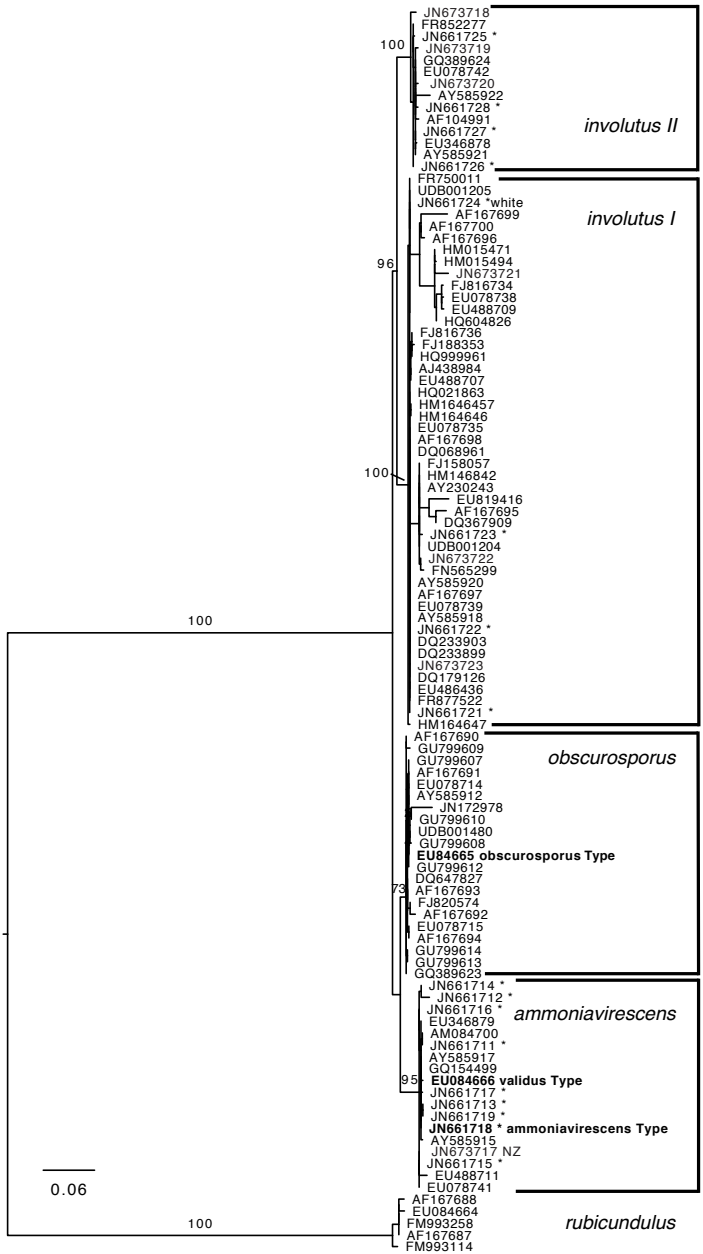
JN673718
FR852277
JN661725 *
JN673719
GQ389624
EU078742
JN673720
AY585922
JN661728 *
AF104991
JN661727 *
EU346878
AY585921
JN661726 *
involutus II
FR750011
UDB001205
JN661724 *white
AF167699
involutus I
AF167700
AF167696
HM015471
HM015494
JN673721
FJ816734
EU078738
EU488709
HQ604826
FJ816736
FJ188353
HQ999961
AJ438984
EU488707
HQ021863
HM1646457
HM164646
EU078735
AF167698
DQ068961
FJ158057
HM146842
AY230243
EU819416
AF167695
DQ367909
JN661723 *
UDB001204
JN673722
FN565299
AY585920
AF167697
EU078739
AY585918
JN661722 *
DQ233903
DQ233899
JN673723
DQ179126
EU486436
FR877522
JN661721 *
HM164647
AF167690
GU799609
GU799607
obscurosporus
AF167691
EU078714
AY585912
JN172978
GU799610
UDB001480
GU799608
EU84665 obscurosporus Type
GU799612
DQ647827
AF167693
FJ820574
AF167692
EU078715
AF167694
GU799614
GU799613
GQ389623
JN661714 *
JN661712 *
JN661716 *
ammoniavirescens
EU346879
AM084700
JN661711 *
AY585917
GQ154499
EU084666 validus Type
JN661717 *
JN661713 *
JN661719 *
JN661718 * ammoniavirescens Type
AY585915
JN673717 NZ
JN661715 *
EU488711
EU078741
AF167688
EU084664
FM993258
AF167687
FM993114
rubicundulus
100
96
100
100
73
95
100
0.06

Fig. 2. *Paxillus involutus* s. str. White variant (coll. & photo S. Kelly).

was proposed in Nova Hedwigia 69(1–2), published in August 1999 (according to the subsequent journal issue). *Paxillus ammoniavirescens* was published in Micologia e Vegetazione Mediterranea 13(2) with the nominal date "1998," but actually printed and distributed to libraries in Spring [northern hemisphere] 1999. Micologia e Vegetazione Mediterranea 13(2) arrived at L on May 20 1999, whereas Nova Hedwigia 69(1–2) arrived on 28 October 1999. This indicates that *P. ammoniavirescens* has priority as the correct name for this taxon.

Paxillus albidulus was described as 'very similar' to *P. involutus* but distinguished from that species by the white pileus, stipe, flesh, and basal mycelium. Pigments were present in the hymenium, and the spore print was 'ochreous to pale brown' (Šutara 1992). At the time *P. albidulus* was described, *P. obscurosporus* and *P. ammoniavirescens* had not yet been recognized as separate species, but the basidiocarp stature and light spore print colour exclude the possibility that *P. albidulus* is a close relative of either of these two species. Hedh et al. (2008) were not able to get sequence data from *P. albidulus*, so its identity will remain unresolved. However, the whitish collection (fig. 2) from the U.K., also lacking pigments in pileus and stipe, fits well into *P. involutus* I (Fig. 1). *Paxillus albidulus* was reported from a predominantly coniferous forest, and as this also fits well with *P. involutus* I, we tentatively place *P. albidulus* in

Fig. 3. *Paxillus ammoniavirescens* (coll. & photo L. Perrone)
Collection: Italy, Sardinia, prov. Olbia-Tempio P., Olbia, Park "Fausto Noce",
11 Jan. 2008, det. L. Perrone & M. Contu (herb. Perrone).

synonymy with *P. involutus*. Recently another pale *Paxillus* variant was studied that also fits into *P. involutus* s. str. (Gelardi et al. 2011); that variant, described as *P. involutus* f. *eburneus* Gelardi et al., has a yellow colouration lacking in the variant from England that we studied.

The most common name used for *Paxillus* sequences deposited in GenBank is *P. involutus*; however, in many cases this is not the correct name. The option for third-party annotations is unfortunately not available, and this case emphasizes the necessity for it (Bidartondo et al. 2008).

Most taxa are widely distributed throughout the northern hemisphere, but unfortunately there are very few data from Asia. *Paxillus involutus* I is recorded from many European countries and North America (from Alaska to Tennessee in the southeast). *Paxillus involutus* II is widespread in Europe (and possibly also in boreal North America) but is better known from introduced *Betula* planted in the urban areas of the North American western states (Washington to California); its occurrence in the Great Smoky Mountains NP (Tennessee) is also confirmed (Fig. 1). *Paxillus obscurosporus* is widespread in Europe and known from China (GenBank DQ647827, as '*P. vernalis*'). Lastly, *P. ammoniavirescens* is known from Europe and introduced into New Zealand. [Southern hemisphere *Paxillus* species have been shown to belong

to a separate lineage and family for which Bresinsky et al. (1999) erected the genus *Austropaxillus* Bresinsky & Jarosch]. Vizzini (2008) identified a Moroccan collection growing under *Eucalyptus* as *P. ammoniavirescens*. After publication of Hahn & Agerer (1999) many more distribution and habitat data have become available for *P. ammoniavirescens* and *P. obscurosporus;* Hahn & Agerer's speculations that these are introduced species in Europe no longer seem to be justified, as both species have been recovered from native habitats within Europe and records from outside Europe are scarce. Hedh et al. (2008, 2009), who recorded all four species from the same county in Sweden, have shown that strains representing all four phylogenetic species were able to form ectomycorrhizas with *Betula* and with *Picea* seedlings, but not all tested strains succeeded in forming mycorrhizas and the success rate among strains varied greatly as well. How the strains behave under natural conditions is, of course, a different question.

Taxonomy

Paxillus ammoniavirescens Contu & Dessì, in Dessì & Contu, Micol. Veg. Medit. 13(2): 123. March 1999 ["1998"]. Fig. 3

Holotype: IB, 21 Nov. 1997, P. Dessì; GenBank nrITS JN661718.

= *Paxillus validus* C. Hahn, in Hahn & Agerer, Nova Hedwigia 69(1–2): 261. August 1999.
Holotype: M, 8 Oct. 1997, C. Hahn, CH 243-97; GenBank nrITS EU84666.

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